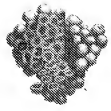

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Cardiac Stem Cells in Brown Adipose Tissue Express CD133 and Induce Bone Marrow Nonhematopoietic Cells to Differentiate into Cardiomyocytes

Yoshihiro Yamada, Shin-ichiro Yokoyama, Xiang-Di Wang, Noboru Fukuda and Nobuyuki Takakura

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Cardiac Stem Cells in Brown Adipose Tissue Express CD133 and Induce Bone Marrow Nonhematopoietic Cells to Differentiate into Cardiomyocytes

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Key Words. AC133 • Adult bone marrow • Myogenesis • Adipogenesis

ABSTRACT

Recently, there has been noteworthy progress in the field of cardiac regeneration therapy. We previously reported that brown adipose tissue (BAT) contained cardiac progenitor cells that were relevant to the regeneration of damaged myocardium. In this study, we found that CD133-positive, but not c-Kit- or Sca-1-positive, cells in BAT differentiated into cardiomyocytes (CMs) with a high frequency. Moreover, we found that $CD133^+$ brown adipose tissue-derived cells (BATDCs) effectively induced bone marrow cells (BMCs) into CMs. BMCs are considered to have the greatest potential as a source of CMs, and two sorts of stem cell populations, the MSCs and hematopoietic stem cells (HSCs), have been reported to differentiate into CMs; however, it has not been determined which population is a better source of CMs. Here we show that CD133-positive BATDCs induce BMCs into CMs, not through cell fusion but through bivalent cation-mediated cell-to-cell contact when cocultured. Moreover, BMCs induced by BATDCs are able to act as CM repletion in an in vivo infarction model. Finally, we found that $CD45^-CD31^-CD105^+$ nonhematopoietic cells, when cocultured with BATDCs, generated more than 20 times the number of CMs compared with $lin^-c\text{-}Kit^+$ HSCs. Taken together, these data suggest that CD133-positive BATDCs are a useful tool as CM inducers, as well as a source of CMs, and that the nonhematopoietic fraction in bone marrow is also a major source of CMs. STEM CELLS *2007;25:1326–1333*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Cell transplantation and gene transfer are two of the foremost therapies with potential for regenerating damaged cardiomyocytes (CMs) and enabling revascularization. Embryonic stem cells, skeletal myoblasts, and c-kit- or Sca-1-positive cardiac stem cells (CSCs) in heart tissue have all been reported as candidates for the replacement of CMs; however, each is associated with problems, including those involving allergenic, etiologic, and arrhythmic issues.

Bone marrow cells (BMCs) are a well-known source of various kinds of stem cells. BMCs can contribute to the regeneration of various tissues, and their clinical application is easier than that of other candidates. Recent studies have suggested that CMs might also originate from BMCs, which are composed of at least two cell populations. Among these are hematopoietic cells (HCs) [1, 2] and mesenchymal cells [3–5], both of which have been suggested as sources of CMs. Nevertheless, the ability of the former to differentiate into CMs is by no means clear. Several studies have demonstrated that hematopoietic stem cells (HSCs) can induce myogenic repair by their capacity to differentiate into CMs [1, 2]. However, two other studies, which used an in vivo infarction model, reported that HSCs were incapable of differentiating into CMs [6, 7]. Moreover, Alvarez-Dolado et al. demonstrated that bone marrow (BM)-derived CMs were observed at low frequency and were generated by cell fusion with donor $CD45^+$ HCs [8]. Taken together, the results from these various studies suggest that it remains to be resolved whether plasticity of HSCs truly occurs and which population will prove to be the best source for the repair of damaged cardiac tissue. Moreover, specific molecular cues for the differentiation of CMs from BMCs have not been identified. This lack of knowledge means that, at present, it is difficult to effectively induce immature cells into CMs.

In this study, we show that CD133 is a CSC marker in brown adipose tissue-derived cells (BATDCs), which had been identified previously as a source of CSCs [9]. CD133 was first isolated from neuroepithelial stem cells as mouse Prominin-1 [10]; subsequently, it was reported that $CD34^+$ HSCs isolated from fetal liver, BM, and cord blood expressed CD133 (AC133) [11]. Moreover, CD133 antigen expression was detected in

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undifferentiated cells, including endothelial progenitor cells [12], fetal brain stem cells [13], embryonic epithelium [14], prostatic epithelial stem cells [15], myogenic cells [16], and certain cancer stem cells, such as retinoblastoma [17] and medulloblastoma [18]. These results indicated that CD133 might be an antigen common to several stem cells, including CSCs, in brown adipose tissue (BAT). Second, we have shown that BMCs could differentiate with high efficiency into CMs by coculturing with BATDCs. In this induction system, we have demonstrated that bivalent cation-mediated cell-to-cell contact was critical for the differentiation of BMCs into CMs, and we have also investigated cadherin-mediated cell contact. Finally, we have demonstrated that in the bone marrow (BM) population, $CD45^-CD31^-CD105^+$ non-HCs could effectively differentiate into CMs, in contrast to the $lin^-c\text{-}Kit^+$ HSCs.

MATERIALS AND METHODS

Cell Preparation and Flow Cytometry

BAT was dissected from the interscapular region of postnatal day 1 (P1) to P7 neonates of C57BL/6 mice. BAT was dissociated by DispaseII (Roche Diagnostics, Mannheim, Germany, http://www.roche-applied-science.com), drawn through a 23-gauge needle, and prepared as a single-cell suspension, as previously reported [9]. BMCs were harvested from femurs and tibias of green fluorescent protein (GFP) transgenic mice (green mice) as previously described [19]. The cell-staining procedure for the flow cytometry was also as previously described [20]. The monoclonal antibodies (mAbs) used in immunofluorescence staining were anti-CD45, -ter119, -CD31, -CD29, -c-kit, -Sca1, -CD105, and -CD133 mAbs (BD Pharmingen, San Diego, http://www.bdbiosciences.com/pharmingen). All mAbs were purified and conjugated with fluorescein isothiocyanate, phycoerythrin (PE), biotin, or allophycocyanin (APC). Biotinylated antibodies were visualized with PE-conjugated streptavidin (BD Pharmingen) or APC-conjugated streptavidin (BD Pharmingen). Cells were incubated for 5 minutes on ice with CD16/32 (FcγIII/II Receptor) (1:100) (Febklok; BD Pharmingen) prior to staining with primary antibody. Cells were incubated in 5% fetal calf serum (FCS)/phosphate-buffered saline (PBS) (washing buffer) with primary antibody for 30 minutes on ice and washed twice with washing buffer. Secondary antibody was added, and the cells were incubated for 30 minutes on ice. After incubation, cells were washed twice with, and suspended in, the washing buffer for fluorescence-activated cell sorting (FACS) analysis. The stained cells were analyzed and sorted with an EPICS Elow cytometer (Beckman Coulter, San Jose, CA, http://www.beckmancoulter.com). The sorted cells were added to 24-well dishes (Nunc, Roskilde, Denmark, http://www.nunchrand.com), precoated with 0.1% gelatin (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com), and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and 10^{-5} M 2-mercaptoethanol at 37°C in a 5% CO_2 incubator.

Immunohistochemistry

Immunohistochemical analyses of tissue sections and culture dishes and the tissue fixation procedure were performed as previously described [9, 20]. The fixed specimens were embedded in Optimum Cutting Temperature (OCT) compound (Sakura Finetechnical, Tokyo, http://www.sakuraus.com) and sectioned at 7 μm. Anti-sarcomeric actin (anti-SA) (clone 5C5; Sigma-Aldrich), anti-GATA-4 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.sebt.com), -MEF2C (Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com), -connexin43 (Sigma-Aldrich), -atrial natriuretic factor (ANF) (Santa Cruz Biotechnology), and -GFP antibodies (Santa Cruz Biotechnology) for tissue sections, and anti-SA, -cardiac troponin T (Santa Cruz Biotechnology), -cardiac troponin I (clone 13–11; Neomarkers, Fremont, CA, http://www.lab vision.com) [21–23], -GATA-4, -MEF2C, and -pan-cadherin (Sigma-Aldrich) antibodies for CM culture dishes were used in this assay. In brief, anti-SA was developed with Alexa Fluor 488-, 546-, or 633-conjugated goat anti-mouse IgM (Molecular Probes Inc., Eugene, OR, http://probes.invitrogen.com); anti-pan-cadherin was developed with 546-conjugated goat anti-mouse IgG (Molecular Probes); and anti-cardiac troponin T, cardiac troponin I, -MEF2C, -GATA-4, -connexin43, and -ANF antibodies were developed with Alexa Fluor 488-, 546-, or 633-conjugated goat anti-rabbit IgG (Molecular Probes). Nuclear staining was performed with 4,6-diamidino-2-phenylindole or TOPRO3 (Molecular Probes). Finally, the sections and dishes were observed using an Olympus IX-70 microscope equipped with UPlanFI 4/0.13 and LCPlanFI 20/0.04 dry objective lenses (Olympus, Tokyo, http://www.olympus-global.com). Images were acquired with a CoolSnap digital camera (Roper Scientific, Tokyo, http://www.roperscientific.com). In all assays, an isotype-matched control Ig was used as a negative control, and it was confirmed that the positive signals were not derived from nonspecific background. Images were processed using Adobe Photoshop 7.0 software (Adobe Systems Inc., San Jose, CA, http://www.abode.com).

Transmission Electron Microscopy

Cells were washed in phosphate buffer and fixed with 2% glutaraldehyde and 1% paraformaldehyde in PBS. Samples were post-fixed with 1% osmium in PBS, rinsed, dehydrated, and embedded in araldite (DAKO, Glostrup, Denmark, http://www.dako.com). Then, samples were cut with a diamond knife and examined under a Jeol 100CX (Tokyo, http://www.jeol.co.jp) electron microscope.

Cell Coculture

BATDCs and BMCs were prepared as described above. In situations where $CD133^+$ BATDCs and BMCs were cocultured in contact conditions, 2×10^4 $CD133^+$ BATDCs were plated per well of a 24-well plate and cultured for 10 days before being fixed with 0.5% paraformaldehyde for 15 minutes at room temperature. Fixed cells were washed extensively, first with PBS and then with DMEM/10% FCS. After washing, 1×10^5 BM mononuclear cells (BMMNCs), 1×10^4 $lin^-c\text{-}Kit^+$ HSCs, or 1×10^4 $CD45^-ter119^-CD31^-CD105^+$ MSCs from GFP mice were cultured with fixed BATDCs, as described above, for 10 days and then stained with anti-SA (Sigma-Aldrich), -GATA-4 (Santa Cruz Biotechnology), -MEF-2C (Cell Signaling Technology), and -GFP (MBL International Corp., Nagoya, Japan, http://www.mblintl.com) antibodies. Under separate conditions, $CD133^+$ BATDCs from ROSA26 mice were cocultured on 0.4-μm cell culture inserts (Becton, Dickinson and Company, San Jose, CA, http://www.bd.com) with BMMNCs from green mice in 0.1% gelatin-coated dishes (Becton Dickinson) for 14 days and then stained with anti-SA (Sigma-Aldrich) and anti-GATA-4 (Santa Cruz Biotechnology).

To analyze the diverse signaling between BATDCs and BM-derived cells, we used an anti-E-cadherin antibody (ECCD-1; Calbiochem, La Jolla, CA, http://www.emdbiosciences.com), E-cadherin-Fc, N-cadherin-Fc, and R-cadherin-Fc (all purchased from R&D Systems Inc., Minneapolis, http://www.rndsystems.com).

Reverse Transcription-Polymerase Chain Reaction Analysis

The RNeasy Mini kit (Qiagen, Hilden, Germany, http://www1.qiagen.com) was used for isolation of total RNA from BAT and BMCs. Total RNA was reverse transcribed using the reverse transcription-polymerase chain reaction (RT-PCR) kit (Clontech, Palo Alto, CA, http://www.clontech.com). The cDNA was amplified using Advantage Polymerase Mix (Clontech) in a GeneAmp PCR system, model 9700 (PerkinElmer Life and Analytical Sciences, Norwalk, CT, http://www.perkinelmer.com), by 40–50 cycles. The sequences of the gene-specific primers for RT-PCR were as follows: 5′-α-myosin heavy chain (MHC), TGTCTGCTCTC-CACCGGGAAAATCT; 3′-α-MHC, CATGGCCAATTCTT-GACTCCCATGA; 5′-β-MHC, AACCCACCCAAGTTCGACA ATG ATCG; 3′-β-MHC, CCAACTTTCCTGTTGCCCAAAA ATG; 5′-α-skeletal actin, GGAGATTGTGCGCGACATC AAAGAG; 3′-α-skeletal actin, CTGGTTCCTCCAATGGGA TATCTTC; 5′-α-cardiac actin, TGTGTTACGTCGCC CTGGATTTTGA; 3′-α-cardiac actin, TTGCTGATCCACATIT

GCTGGAAGG; 5′-myosin light chain (MLC)-2a, AGCAGGCA-CAACGTGGCTCTTCTAA; 3′-MLC-2a, CCTGGGTCAT-GAGAAGCTGCTTGAA; 5′-MLC-2v, ATGGCACCTTTGTTTGC-CAAGAAGC; 3′-MLC-2v, CCCTCGGGATCAAACACCTTAATG; 5′-GATA4, GAGTGAGTCAATTGTGGGGCCATGT; 3′-GATA4, TGCTGCTAGTGGCA TTGCTGGAGTT; 5′-glyceraldehyde-3-phosphate dehydrogenase (G3PDH), TGAAGGTCGGTGT-GAACGGATTTGGC; 3′-G3PDH, CATGTAGGCCATGAG GTC-CACCAC; 5′-lacZ, GCGTTACCCAACTTAATCG; 3′-lacZ, TGTGAGCGAGTAACAACC; 5′-GFP, TACGGCAAGCTGAC-CCTGAA; 3′-GFP, TGTGATCGCGCTTCTCGTTG. Each cycle consisted of denaturation at 94°C for 30 seconds and annealing/extension at 70°C for 4 minutes except lacZ and GFP. For lacZ and GFP, each cycle consisted of denaturation at 94°C for 30 second, annealing at 60°C for 1 minute, and extension 72°C for 1 minute.

Myocardial Infarction and Cell Implantation and Echocardiography

BMCs derived from GFP transgenic Sprague-Dawley rats [24] at 2 months old were cocultured for 5 days with CD133$^+$ BATDCs taken from wild-type Sprague-Dawley rats at 3 days, and then the GFP-positive fraction was sorted using an EPICS flow cytometer and implanted into the heart. Myocardial infarctions (MIs) were induced in female Sprague-Dawley rats at 2 months of age as described previously [9]. After verifying the MIs, 10 rats were injected with 2×10^5 cells each, in five opposite regions bordering the infarct, and then sacrificed after 30 days. At each time interval, sham-operated rats were injected with saline as controls. Under ketamine (Dainippon Pharmaceutical, Osaka, Japan, http://www.ds-pharma.co.jp) anesthesia, echocardiography was performed at 29 days. From M mode tracings, left ventriculus end-diastolic and systolic diameter and wall thickness were obtained, and then the percentage of fractional shortening was calculated. Echocardiographic acquisition and analysis were performed by an echocardiographer blinded to treatment group. Results represent the mean of five separate experiments. Mortality was lower, but not significantly different, in the treated rats, averaging 35% in all groups. Protocols were approved by the institutional review board.

Fluorescence In Situ Hybridization Staining

After staining of CMs with anti-SA antibody as described above, we performed fluorescence in situ hybridization (FISH) to determine the presence of rat X and Y chromosomes (Cambio, Cambridge, U.K., http://www.cambio.co.uk) according to the manufacturer's instructions. A digestion with proteinase K (DAKO) (20 μg/ml at 37°C, 5 minutes) was added at the beginning of the FISH protocol. Nuclear staining was performed with TOPRO3 (Molecular Probes). Pictures were taken by confocal microscope (LSM510; Carl Zeiss MicroImaging, Inc., Göttingen, Germany, http://www.zeiss.com).

Results

Surface Phenotype of CM Progenitors in BAT

A putative stem cell population has recently been identified in adipose tissue [25]. This cell population expressed multiple CD marker antigens, such as CD29, CD44, CD90, and CD105, similar to those observed on MSCs. We previously reported that BATDCs included cells that are able to differentiate into CMs [9], and another group has demonstrated the capacity of adipose tissue-derived mesenchymal cells to differentiate into CMs [26]. Other reports have indicated that cardiac progenitor cells in the adult heart expressed c-Kit and Sca-1, which were first established as HSC markers [21, 27]. Therefore, using flow cytometric analysis, we analyzed several stem cell markers and multiple CD markers on cells from BATDCs, which can differentiate into CMs. First, we checked for ter119 (erythrocyte marker), CD45 (LCA) (panleukocyte marker), and CD31 (endothelial marker). Among CD45$^-$ter119$^-$CD31$^-$ cells (nonhematopoietic, nonendothelial cells), c-Kit, Sca-1, and CD133 expression was observed on 1.4%, 16.6%, and 3.5% of total BATDCs, respectively (Fig. 1A). To clarify which populations have a high potential to produce CMs, we cultured a CD45$^-$ter119$^-$CD31$^-$ subpopulation fractionated by c-Kit, Sca-1, or CD133 expression. Among them, CD133-positive BATDCs differentiated into CMs with a higher incidence compared with cells in other fractions and CD29-positive (CD45$^-$ter119$^-$CD31$^-$) cells, which we had previously reported as a CM-enriched population (Fig. 1C). Indeed, approximately 50% of adherent cells from cultured CD133-positive cells among CD45$^-$ter119$^-$CD31$^-$ cells differentiated into SA$^+$GATA-4$^+$ (Fig. 1B, a and b, 1C, a and b), cardiac-troponin T$^+$GATA-4$^+$ (Fig. 1B, c and d), and cardiac-troponin I$^-$MEF2C$^+$ (Fig. 1B, e and f). Furthermore, electron microscopic analysis indicated that these cells had cellular structures typical of CMs, such as an organized sarcomere with typical cross-strain, developed Z-bands, long mitochondria in the cytoplasm, and centrally positioned nuclei (Fig. 1B, g). To provide additional evidence that CD133$^+$ BATDCs have the phenotype of CMs, we performed pharmacological studies (supplemental online data). The calcium antagonist verapamil slowed the beating rate, and the β-agonist isoproterenol induced a dose-dependent increase of the spontaneous contraction rate; however, the β-adrenergic antagonist propranolol reversed the isoproterenol-induced acceleration. These results also indicated that CD133$^+$ BATDCs have the functional character of CMs.

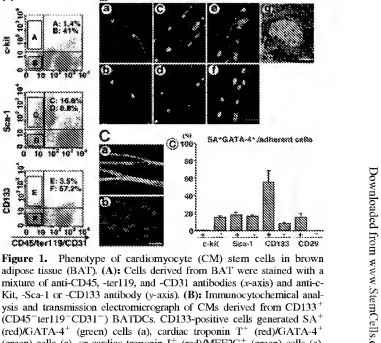

Figure 1. Phenotype of cardiomyocyte (CM) stem cells in brown adipose tissue (BAT). **(A):** Cells derived from BAT were stained with a mixture of anti-CD45, -ter119, and -CD31 antibodies (*x*-axis) and anti-c-Kit, -Sca-1 or -CD133 antibody (*y*-axis). **(B):** Immunocytochemical analysis and transmission electromicrograph of CMs derived from CD133$^+$ (CD45$^-$ter119$^-$CD31$^-$) BATDCs. CD133-positive cells generated SA$^+$ (red)/GATA-4$^+$ (green) cells (a), cardiac troponin T$^+$ (red)/GATA-4$^+$ (green) cells (c), or cardiac troponin I$^-$ (red)/MEF2C^{++} (green) cells (e). Nuclear staining by TOPRO3 in a, c, and e is shown in b, d, and f, respectively. Notably, well-organized sarcomeres, Z-band, and a large number of mitochondria were observed in CD133$^+$ BATDCs. Scale bar = 10 μm (f) and 1 μm (g). **(C):** a, Existence of SA-positive (green) and GATA4-positive (red) CMs in the culture of CD133+ BATDCs. b, Nuclear staining with 4,6-diamidino-2-phenylindole in the same field as shown in panel a. Note that this low-power field view clearly showed that approximately 50% of adherent cells are CMs. Scale bar = 20 μm. c, Quantitative evaluation of differentiation potential for CMs from each fraction of BATDCs as indicated. The same number of cells (1×10^4) of each fraction was cultured. Note that c-Kit$^+$ (CD45$^-$ter119$^-$CD31$^-$) cells could not differentiate into CMs, and CD133$^+$ (CD45$^-$ter119$^-$CD31$^-$) cells were the most effective at differentiating into CMs.

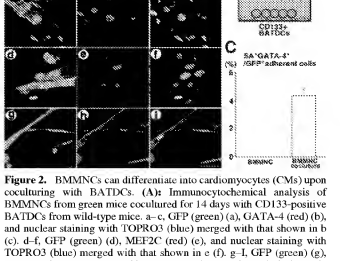

Figure 2. BMMNCs can differentiate into cardiomyocytes (CMs) upon coculturing with BATDCs. **(A):** Immunocytochemical analysis of BMMNCs from green mice cocultured for 14 days with CD133-positive BATDCs from wild-type mice. a–c, GFP (green) (a), GATA-4 (red) (b), and nuclear staining with TOPRO3 (blue) merged with that shown in b (c). d–f, GFP (green) (d), MEF2C (red) (e), and nuclear staining with TOPRO3 (blue) merged with that shown in e (f). g–I, GFP (green) (g), SA (red) (h), and GATA-4 (blue) merged with that shown in h (i). Scale bar = 5 μm (i). **(B):** Schematic presentation of coculturing BMMNCs from green mice with CD133-positive BATDCs from wild-type mice. **(C):** Quantitative evaluation of differentiated SA- and GATA4-positive CMs among adhering GFP-positive BM-derived cells. Data for BMMNC cocultured with CD133+ BATDCs and BMMNCs cultured alone are displayed. Results represent the mean of five independent experiments. Abbreviations: BATDC, brown adipose tissue-derived cell; BMC, bone marrow cell; BMMNC, bone marrow mononuclear cell; GFP, green fluorescent protein.

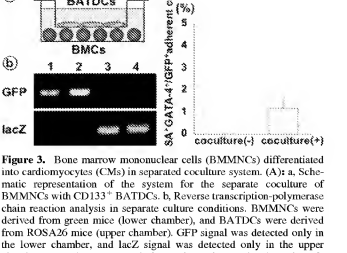

Figure 3. Bone marrow mononuclear cells (BMMNCs) differentiated into cardiomyocytes (CMs) in separated coculture system. **(A):** a, Schematic representation of the system for the separate coculture of BMMNCs with CD133$^+$ BATDCs. b, Reverse transcription-polymerase chain reaction analysis in separate culture conditions. BMMNCs were derived from green mice (lower chamber), and BATDCs were derived from ROSA26 mice (upper chamber). GFP signal was detected only in the lower chamber, and lacZ signal was detected only in the upper chamber. Lane 1, BMMNCs before culture (lower chamber); lane 2, BMMNCs after 14 days of culture (lower chamber); lane 3, BATDCs before culture; lane 4, BATDCs after 14 days of culture. **(B):** Quantitative evaluation of the differentiation potential for CMs from BMMNCs in separate culture conditions. Upon coculturing with BATDCs (coculture[+]), approximately 1.7% of adhering GFP-positive bone marrow cells differentiated into SA- and GATA4-positive CMs. CM development could not be observed when BMMNCs were cultured alone (coculture[−]). Results represent the mean of five independent experiments. Abbreviations: BATDC, brown adipose tissue-derived cell; BMC, bone marrow cell; GFP, green fluorescent protein.

BATDCs Effectively Induce CM Production from BMCs

A previous in vitro study showed that although BMCs were a source of CMs, they could not spontaneously differentiate into CMs; however, differentiation could be induced with 5-azacytidine, a DNA-hypomethylating agent [4]. It is thought that BMCs can differentiate into CMs upon adequate environmental molecular cues. As CD133$^+$ BATDCs differentiate into CMs spontaneously, this suggests that CD133$^+$ BATDCs produce molecules that induce the differentiation of CD133$^+$ BATDCs into CMs by an autocrine loop. Therefore, to test this ability, we cultured BMMNCs with BATDCs and observed whether or not, under these conditions, BMMNCs could differentiate into CMs. At first, CD133$^+$ cells were sorted from BAT by FACS and cultured on 0.1% gelatin-coated dishes. After 1 week, BMMNCs derived from green mice that express GFP ubiquitously in their tissues [28] were cocultured in direct contact with CD133$^+$ BATDCs, as shown in Figure 2B. By coculturing BMMNCs with CD133-positive BATDCs, nuclear-located GATA-4-positive (Fig. 2A, a–c), MEF2C-positive (Fig. 2A, d–f), and SA-positive (Fig. 2A, g–i) contracting cells were produced among the GFP-positive BMMNCs. However, the SA-positive and GATA-4-positive cells were not observed when BMMNCs were cultured alone under the same conditions (Fig. 2C).

BMMNCs with BATDCs Differentiated into CMs Without Fusion Mechanism

Recently, it has been suggested that cell fusion is the main mechanism that contributes to the development or maintenance of cardiac muscle and neuron regeneration [8, 29]. To exclude the possibility of cell fusion in our experiment, we cultured BMMNCs from green mice with BATDCs from ROSA26 mice, which express LacZ ubiquitously in their tissues, and separated the cells from these two different origins with a 0.4-μm-pore membrane (Fig. 3A, a). In this separating coculture system, SA$^+$GATA-4$^+$ CMs were also produced from BMMNCs when cocultured with BATDCs (Fig. 3B). Moreover, with PCR analysis, GFP-positive signals were detected only in the BMMNC layer, and lacZ-positive signals were detected only in the BATDCs layer from the cells before and after culturing (Fig. 3A, b). Therefore, we concluded that the development of CMs from BMMNCs was not dependent on cell fusion with BATDCs.

Cell Contact Mediated by Bivalent Cation Is Critical for the Differentiation of BMMNCs into CMs

We found that BMMNCs could differentiate into CMs under conditions in which the BMMNCs were cultured in direct or indirect contact with BATDCs; however, differentiation of BMMNCs into CMs was promoted more effectively by direct cell-to-cell contact between BMMNCs and BATDCs than under separated culture conditions (Figs. 2B, 3B). Indeed, 520 $\pm$ 40 versus 105 $\pm$ 18 SA$^+$GATA4$^+$ CMs were generated from 1×10^5 BMMNCs under direct and indirect cocultures, respectively. To exclude the possibility of cell fusion in the contact coculture system, we used paraformaldehyde-fixed cultured CD133$^+$ BATDCs, which cannot fuse with other cells but have an intact cell surface. After coculturing with fixed BATDCs, some GFP$^+$ BMMNC-derived cells (Fig. 4A, a) expressed cardiac-specific antigens, such as SA (Fig. 4A, b and c), GATA-4, and cardiac troponinT (data not shown). Moreover, these cells also had the typical features of CMs, confirmed by electron microscopic analysis (Fig. 4A, f).

Cadherin-mediated calcium-dependent cell-to-cell contact is widely believed to be involved in the regulation of the diverse signaling process for cell differentiation [30]. Therefore, we elucidated a potential role for calcium-dependent cell-to-cell contact in our coculture system. First, BMMNCs were incubated with the calcium chelator EDTA or EGTA on fixed BATDCs. This treatment abolished the adhesion of BMMNCs to fixed, differentiated BATDCs. By the suppression of cell adhesion of BMCs to differ-

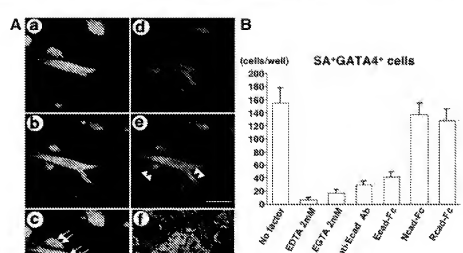

Figure 4. Differentiation of bone marrow mononuclear cells (BMMNCs) into cardiomyocytes (CMs) is mainly induced by bivalent cation-mediated cell contact with brown adipose tissue-derived cells (BATDCs). **(A):** Immunocytochemical staining of CMs from BMMNCs cocultured with fixed BATDCs for 14 days. Anti-GFP (green) (a and c), anti-SA(red) (d and e), and anti-pan-cadherin (blue) (b, c, and e) Abs were used in this assay. Arrows indicate SA^+GFP^+ cells interacting with SA^+GFP^- cells (arrowheads) through cadherin expression. f, Transmission electron micrographic analysis showed that BM-derived CMs had well-organized sarcomeres, Z-band, and a large number of mitochondria. Scale bar = 10 μm (e) and 1 μm (f). **(B):** Quantitative evaluation of differentiated CMs from BMMNCs in the presence or absence of EGTA, EDTA, or 100 μg/ml neutralizing Ab of Ecad, Ecad-Fc, Ncad-Fc, or Rcad-Fc when cocultured with fixed BATDCs. Adherent SA-positive and GATA-4-positive cells were counted in each culture condition. Results represent the mean of five independent experiments. Abbreviations: Ab, antibody; Ecad, E-cadherin; Ncad, N-cadherin; Rcad, R-cadherin.

entiated BATDCs (fixed), differentiation of BMCs into CMs was almost completely suppressed (Fig. 4B). Next, we examined which, out of E-cadherin (ECad), NCad, or RCad, was affected in this mechanism. We found that $CD133^+$ BATDCs abundantly expressed Ecad, and BMCs expressed Ecad and Ncad, as confirmed by PCR analysis (data not shown). To evaluate the effect of cadherins in this culture system, we added neutralizing antibody (Ab) against Ecad (anti-Ecad Ab) or soluble cadherin, such as Ecad-Fc, Ncad-Fc, or Rcad-Fc. Among these materials, anti-Ecad Ab and Ecad-Fc effectively inhibited the differentiation of BMMNCs into CMs. Taken together, these data suggest that Ecad-mediated cell-to-cell contact is important for the differentiation of BMMNCs into CMs.

Educated BMMNCs Contributed to CM Regeneration

The data above clearly indicate that CD133-positive BATDCs effectively induced CM production from BMCs. Next, we evaluated the length of time required for BMMNCs to become committed to CM lineage when cultured with BATDCs. For this purpose, we attempted to coculture BMMNCs from green rats expressing GFP with BATDCs for 1 to 10 days and harvested GFP-positive cells at intervals from the culture. GFP-positive cells were then sorted and cultured alone for an additional 14 days (Fig. 5A, a). In this experiment, we found that 5 days was enough for commitment of BMMNCs into CM lineage. Sorted GFP-positive cells did not express SA and GATA-4 (data not shown); however, they started to display contractile activity after 7 days of culturing. After 14 days of culturing, we found that approximately 10% of GFP-positive adherent cells differentiated into SA-positive and MEF2C-positive cells (Fig. 5A, b–d). To examine cardiac-specific genes and transcription factors in detail, we performed RT-PCR analysis and revealed that the expression of α-MHC, β-MHC, α-skeletal actin, α-cardiac actin, MLC-2v, and GATA4 was detected (Fig. 5A, e). Interestingly, this phenotype was similar to that of CMs derived from BATDCs [9].

Next, to determine whether BMMNCs exposed to BATDCs could effectively contribute to the regeneration of the heart, we injected the exposed BMMNCs into the hearts of rats after the induction of an acute MI. At first, we cocultured GFP-positive BMMNCs with CD133-positive BATDCs for 5 days, purified the GFP-positive cells by FACS, and injected the cells into the hearts of experimental MI rats at each of five sites at the border of the infarcted tissue. As a control, infarcted hearts were injected either with equal volumes and numbers of nonexposed (naive) BMMNCs or with saline. First, we revealed that donor-derived, exposed GFP-positive and SA- or ANF-positive cells were detected in abundance in the infarct border zone (Fig. 5B, a and d; 16.8% ± 2.1% of total cardiomyocytes in one field), but there were 15-fold fewer after injecting nonexposed GFP-positive cells (1.1% ± 0.4%). Hearts injected with exposed BMMNCs expressed SA-positive CMs, which also expressed connexin43 (Fig. 5B, a–c), and ANF (Fig. 5B, d–f). This indicated that transplanted BMMNCs formed gap junctions and intercalated disks and secreted CM-specific factor. Moreover, the assessment of cardiac function by echocardiography revealed that the hearts injected with exposed BMMNCs showed improved contractions of movement of the infarcted anterior walls and reduced left ventricular remodeling compared with the hearts injected with naive BMMNC or saline (Table 1). To exclude the possibilities of cell fusion in this model, we performed FISH staining with chromosome X- and Y-specific paint and revealed that educated BMMNCs (e-BMCs) implanted from male rats into female rats formed CM stained only with XY chromosome, not XXXY (Fig. 5C, a and b). This indicated that in vivo cardiac differentiation of BMMNCs was not induced by the fusion mechanism.

Nonhematopoietic Cells in the BM Are a Major Source of CM

Two candidates for the ability to regenerate myocardial tissue are HSCs and nonhematopoietic MSCs, as previously reported [1–4]. However, a recent report has indicated that the

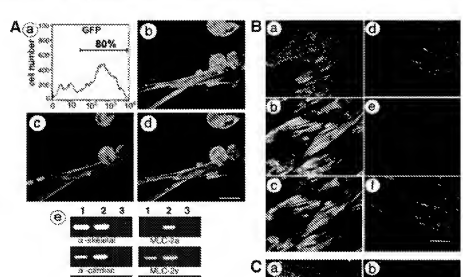

Figure 5. Bone marrow mononuclear cells (BMMNCs) cocultured with brown adipose tissue-derived cells (BATDCs) can contribute to cardiac regeneration. **(A):** Phenotype analysis of cardiomyocytes (CMs) derived from BMMNCs. a, After BMMNCs from green rats were cocultured for 5 days with BATDCs from wild-type rats, FACS analysis showed that 80% of cells were GFP-positive. b–d, Isolated GFP-positive fraction as indicated in panel a were cultured again in the absence of BATDCs for 14 days. Among adherent GFP-positive cells (green) (b), approximately 9.7% showed SA staining (red). d, MEF2C staining (blue) merged with that shown in b and c. e, Reverse transcription-polymerase chain reaction analysis of several CM-specific genes in CMs produced from BMMNCs. Lane 1, cells harvested from the conditions described in b–d (educated BMCs [e-BMCs]); lane 2, CMs from neonatal mice as a positive control; lane 3, total RNA used in lane 1 without reverse transcription. Scale bar = 5 μm. **(B):** Cells obtained as described in **(A)** were injected into infarcted heart, and the tissue distribution of various marker molecules was determined. a–c, GFP (green) (a), SA (blue) (b), and connexin43 (red) merged with that shown in a and b (c). d–f, GFP (green) (d), ANF (red) (e), and nuclear staining with TOPRO3 (blue) merged with that shown in d and e (f). Scale bar = 5 μm (f). **(C):** Fluorescence in situ hybridization staining in implanted site of CM. a, Section was stained with anti-SA (green) and TOPRO3 (blue). b, Serial section of panel a. Green indicates X chromosome, and red indicates Y chromosome. Nuclear staining was performed with TOPRO3 (blue). Cells expressing X and Y chromosome in the nuclei indicate that these cells were derived from e-BMCs and did not fuse with host CM expressing only X chromosome. Scale bar = 5 μm. Abbreviations: G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein.

Table 1. Effect of transplanted cells on myocardial performance

	Sham-operated	e-BMCs	Non-e-BMCs	Saline
Echocardiography				
Chamber diameter (mm)	6.11 ± 0.30	7.39 ± 0.70[a,b]	8.09 ± 0.77	10.8 ± 0.58
Viable WT (mm)	1.81 ± 0.17	1.71 ± 0.21[b]	1.70 ± 0.22	1.66 ± 0.13
Infarcted WT (mm)	1.80 ± 0.16	1.35 ± 0.24[a,b]	1.01 ± 0.32	0.75 ± 0.20
% Fractional shortening (%)	78.9 ± 2.1	41.9 ± 3.7[a,b]	33.1 ± 4.3	21.6 ± 2.6

The echocardiography revealed that the experimental infarct group injected with bone marrow mononuclear cells (BMMNCs) cocultured with brown adipose tissue-derived cells (BATDCs) (BMMNCs exposed to BATDCs; e-BMCs) had improved fractional shortening and reduced left ventricular internal dimension at end-diastole compared with the naïve BMMNCs (non-e-BMC)-injected group or the saline-injected group.
[a]Statistically significant difference from bone marrow ($p < .02$).
[b]Statistically significant difference from saline ($p < .01$).
Abbreviations: e-BMC, educated bone marrow cell; WT, wall thickness.

former source of CMs was generated by fusion with donor $CD45^+$ cells, suggesting that plasticity of HSC might result from the fusion of HSCs with resident cells [9]. To reveal which stem cells were capable of differentiating into CMs, we compared an HSC population and a nonhematopoietic MSC population for their ability to differentiate into CMs when cocultured with BATDCs. We sorted $Lin^-c\text{-}Kit^+$ cells and $CD45^-CD31^-CD105^+$ cells in BM from green mice, as typical of HSC and nonhematopoietic MSC populations, respectively (Fig. 6A), and then cocultured them with CD133-positive BATDCs, as shown in Figure 2B. After 14 days, cultured cells were stained with cardiac-specific antigen, which revealed that $SA^+/GATA\text{-}4^+$ cells were generated from both populations; however, the efficiency for differentiation into CMs from those two populations was quite different. As shown in Figure 6B, the number of $SA^+/GATA\text{-}4^+$ CMs from $CD45^-CD31^-CD105^+$ was over 20 times greater than that from $Lin^-c\text{-}Kit^+$ cells (Fig. 6B). These results suggested that nonhematopoietic MSCs are the major source of CMs in the BM.

DISCUSSION

Previously, we showed that BATDCs possessed cardiac progenitor cells, which contributed to the in vivo regeneration of damaged cardiac tissues [9]. Therefore, we suggested that BATDCs might be one of the prospective sources that could overcome issues associated with the regeneration of CMs. In this

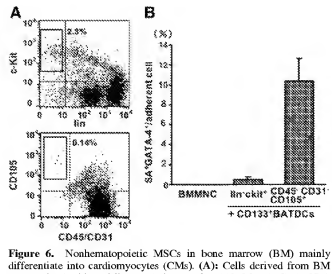

Figure 6. Nonhematopoietic MSCs in bone marrow (BM) mainly differentiate into cardiomyocytes (CMs). **(A):** Cells derived from BM were stained with anti-lineage (mixture of ter119, Mac-1, Gr-1, CD4, CD8, and B220 antibodies) marker or anti-CD31 and anti-CD45 antibodies (*x*-axis) and anti-c-Kit or anti-CD105 antibody (*y*-axis). The percentage of $lin^-c\text{-}Kit^+$ cells and $CD45^-CD31^-CD105^+$ cells in BM are indicated in the box. **(B):** Quantitative evaluation of differentiated SA- and GATA-4-positive CMs from fractionated BMMNCs, as indicated under coculturing with $CD133^+$ BATDCs. Among the adhering BM cells, 10.7% (850 ± 172) and 0.4% (23 ± 3) were $SA^+GATA\text{-}4^+$ cells from $lin^-c\text{-}Kit^+$ and $CD45^-CD31^-CD105^+$ cells, respectively. BMMNCs alone did not produce CMs. Results represent the mean of five independent experiments. Abbreviations: BATDC, brown adipose tissue-derived cell; BMMNC, bone marrow mononuclear cell.

study, we looked for stem cell markers of CMs in BAT. To this purpose, we compared the capacity for differentiation into CMs from fractionated cells based on the expression levels of c-Kit, Sca-1, and CD133. We could not induce CMs from c-Kit-positive cells, and Sca-1 did not notably increase the numbers of cells that differentiated into CMs. However, there was a remarkable difference in favor of CD133-positive over CD133-negative cells in the ability to differentiate into CMs. Previous studies indicated that CD133 is a common stem cell marker, associated with, for example, neural stem cells, HSCs, endothelial progenitor cells (EPCs), epithelial stem cells, and cancer stem cells. Moreover, it was reported that EPCs differentiated into CMs upon coculturing with neonatal CMs [31]. The results from these previous studies, taken alongside those from our present study, strongly suggest that CD133 might be the CSC marker for stem cell sources in adipose and other tissues. CD133-expressing cells in BAT from the interscapular region were relatively abundant in the embryonic and neonatal stage but were reduced in the adult stage (3.5% vs. 0.2%; data not shown). Moreover, BAT does not exist in great abundance in the adult human. Therefore, BAT itself might not have a clinical application for myocardial disease in adult patients. However, using the evidence that BATDCs can differentiate into CMs and CD133-positive cells from BAT can induce BMMNCs into CMs, we might be able to investigate how BMCs can differentiate into CMs at a molecular level.

In this study, we found that when cocultured in vitro with BATDC, BMCs themselves can give rise to CMs effectively without cell fusion. In the coculture system on $CD133^+$ BATDCs, 1×10^5 total BMMNCs, which logically contained about 140 $CD45^-CD31^-CD105^+$ cells, generated 520 SA^+GATA4^+ cells. On the other hand, in the coculture of nonhematopoietic cells from BM with $CD133^+$ BATDCs, 1×10^4 $CD45^-CD31^-CD105^+$ cells generated approximately 850 SA^+GATA4^+ cells. This low efficiency of induction for CMs from $CD45^-CD31^-CD105^+$ cells may have been due to the lack of other adherent cells observed in the culture using total BMMNCs. At present, we could not determine which adherent cells from BMMNCs were important; however, macrophage and/or endothelial cells may play a role in the generation of CMs synergistically with $CD133^+$ BATDCs.

In the coculture system, when cell-to-cell contact coculture conditions were compared with this filter-separated coculture system, the former effectively induced BMCs into CMs. The culture supernatant of cultured BATDCs, even concentrated, induced BMCs into CMs with an efficiency similar to that observed in the separate coculture conditions. These results suggested that there might be two independent ways of inducing CMs from BMCs. One way is to use the fact that secreted factors determine the fate of BMCs into CMs and the other is that membrane proteins do. With respect to the latter, we suggested that cadherins may mediate this crucial cell-to-cell contact. For the physical interaction of cohering cells, cadherins regulate diverse signaling process, such as differentiation, proliferation, and migration. As previously reported, Ecad-mediated cell-cell interaction, among cells that contain primordial germ cell precursors, is essential to directing such cells to the germ cell fate [30]. In the case of CM development, there is no report showing cadherin-dependent cell commitment; however, an important role for the wnt-frizzled pathway, which is closely related to cadherin, in cardiac development was reported previously [32]. Indeed, calcium depletion with EDTA or EGTA, which prevents cadherin-mediated cell-to-cell contact, abolished the adhesion of BMMNCs to CMs. In addition, cadherins are expressed in GFP^+ BMMNC-derived cells and are localized to the sites of cell-to-cell contact between BMMNCs and BAT CMs (Fig. 4A). Among several type of cadherins, we detected that the suppression of Ecad function was effective in inhibiting both the adhesion of BMMNCs to BATDCs and their differentiation into CMs (Fig. 4B). These data suggest a potential role of Ecad in the differentiation of BMMNCs into CMs. However, additional experiments will be necessary to show the molecular mechanisms behind the cell fate decision that involve cell-to-cell contact. Moreover, regarding the possibility of the existence of a secreted factor from BATDCs for the induction of BMCs into CMs, we found that $CD133^+$ cells expressed platelet-derived growth factor (PDGF)-AB (data not shown), and BMCs expressed platelet-derived growth factor receptor α (PDGFR-α) (data not shown). To understand the contribution of the PDGF-PDGFR system, we added neutralizing antibody against PDGFR-α into the coculture of $CD133^+$ BATDCs and BMCs. Some inhibition of the differentiation from BMCs into SA^+GATA4^+ CMs was observed after 14 days of culturing (data not shown). Moreover, we found that BATDCs expressed beneficial cytokines, such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and angiopoietin-1 (data not shown). These and other, unknown factors induced the proliferation and antiapoptotic effect of e-BMCs and contributed to the effective differentiation into SA^+GATA4^+ CMs. With these data, taken together, we confirmed that there might be two processes involved in the effective induction of BMCs into CMs: a secreted protein and cell-to-cell contact mediated by a membrane-binding protein.

With the in vivo infarction model, e-BMCs effectively differentiated into CMs and improved cardiac function. In regard to this point, we found that VEGF and HGF were highly expressed by e-BMCs compared with non-e-BMCs. Moreover, approximately twice the number of $CD31^+$ ECs was observed in the transplanted region of e-BMCs compared with that of non-e-BMCs, and we also found that a small number of e-BMCs incorporated as $CD31^+$ ECs or α-smooth muscle $actin^+$ mural cells (data not shown). These results indicated that the paracrine

effect on neighboring cardiac myocytes and angiogenesis also contributed to the beneficial effects of transplantation.

Furthermore, we found no significant difference in the total number of GFP-positive cells located around the ischemic border zone between BMMNCs exposed to BATDCs (educated BMMNCs) and noneducated BMMNCs; however, SA^+/GFP^+ CMs derived from educated BMMNCs were more than 15 times more abundant in number compared with those from noneducated BMMNCs. Recently, some groups have indicated that $VEGFR\text{-}2^+/VE\text{-}cadherin^-$ primitive cells from embryonic stem cells do not contribute to vascular cells, such as ECs or SMCs. By contrast, differentiated $VE\text{-}cadherin^+$ EPCs contributed to the vascular formation as ECs [33]. These studies suggested that committed immature cells are better sources for vascular or myocardial regeneration than undirected mesodermal-derived stem cells.

In summary, we suggest that CD133-positive cells in BATDCs form an enriched stem cell population, as well as being effective inducers from MSCs in BM to CMs in vitro and in vivo. CD133-positive cells in BATDCs might prove to have potential for the regeneration of CMs.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Cardiac Stem Cells in Brown Adipose Tissue Express CD133 and Induce Bone Marrow Nonhematopoietic Cells to Differentiate into Cardiomyocytes

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